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TOXIC HAZARDS EVALUATION OF FIVE ATMOSPHERIC POLLUTANTS FROM ARMY AMMUNITION PLANTS

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AMRL-TR-77-25

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
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Tetranitromethane	Acute oral toxicity
Ortho nitrotoluene	Acute skin toxicity
Meta nitrotoluene	Subchronic inhalation toxicity
Para nitrotoluene	Acute intravenous

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Acute toxic effects of methyl nitrate, tetranitromethane (TNM) and the ortho, meta, and para nitrotoluenes were determined using rats, mice, guinea pigs and rabbits. Subchronic toxicity of TNM to rats was compared to that of NO_2 and found to be sufficiently different to lead to an inference of different mechanisms for each contaminant.

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SUMMARY

Section I

The acute toxic properties of methyl nitrate, tetranitromethane (TNM) and the ortho, meta, and para nitrotoluenes were investigated. Four hour LC_{50} values were obtained for all compounds using rats and mice. Skin absorption LD_{50} values and skin irritation scores in rabbits were measured for the nitrotoluenes. Oral LD_{50}'s in rats and mice were determined for methyl nitrate and tetranitromethane and intravenous LD_{50}'s for the latter compound.

No toxic effects were noted after exposure to any of the nitrotoluenes by any route of administration. Toxicity of methyl nitrate either by inhalation or ingestion could be ascribed to production of methemoglobin. Methemoglobinemia appeared to be the major toxic result of oral dosing of tetranitromethane, but inhalation or intravenous administration of the compound led to severe pulmonary irritation similar to that caused by nitrogen dioxide (NO_2)

Section II

Simultaneous, 2-week continuous inhalation exposures of rats to TNM and to NO_2 at 4 times the molar concentration of TNM were carried out to test the hypothesis that the toxicity of TNM was quantitatively identical to that of NO_2 at 4 times the concentration. A repeat experiment was carried out at the highest TNM concentration along with one at an NO_2 concentration 5.4 times the TNM level. Results of these experiments showed that the toxic effects of the two contaminants were different qualitatively and quantitatively.

An additional experiment was performed in which the oral toxicity of methyl nitrate to guinea pigs was determined. It demonstrated that the toxicity of this compound is not a function of animal size.

PREFACE

This technical report describes results of investigations into the toxic properties of the ortho-, meta- and para- nitrotoluenes, methyl nitrate, tetranitromethane and nitrogen dioxide. The first phase of the study covering the acute toxicities of all the compounds but nitrogen dioxide was performed from 12 May 1975 to 30 September 1975. The second phase, primarily concerned with subchronic toxicity of tetranitromethane and nitrogen dioxide, took place during the period from 1 February 1976 to 1 August 1976. The work was supported by the US Army under Military Interdepartmental Purchase Request Number 5969 and performed by the University of California, Irvine, Department of Community and Environmental Medicine, Toxic Hazards Research Unit at Wright-Patterson Air Force Base, Ohio under Air Force Contract No. F33615-76-C-5005. K. C. Back, Ph.D., Chief of the Toxicology Branch was the technical monitor for the Aerospace Medical Research Laboratory.

T. Timothy Crocker, M.D., was the principal investigator for the University of California, Irvine. Acknowledgement is made to Allen Hall, III, Maj. USAF, VC, for pathological examination and evaluation of tissues from animals exposed in this study.

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SECTION I

ACUTE TOXICITY OF FIVE ATMOSPHERIC POLLUTANTS FROM ARMY AMMUNITION PLANTS

E. R. Kinkead, J. D. MacEwen and E. H. Vernot

INTRODUCTION

Acute toxicity studies were conducted on five atmospheric pollutants, resulting from the manufacture of munitions, to evaluate the community and environmental health hazard associated with their emission. These studies were undertaken at the request of the US Army as a preliminary step toward establishing criteria for setting environmental or emission standards.

MATERIALS AND METHODS

The group of compounds and the route of administration for each are shown below:

	Route of Administration				
Compound	Oral	Intra-venous	Skin Absorp-tion	Skin Irrita-tion	Inhala-tion
Methyl Nitrate	x				x
Tetranitromethane	x	x			x
o-Nitrotoluene			x	x	x
m-Nitrotoluene			x	x	x
p-Nitrotoluene			x	x	x

The experimental animals were fasted for a minimum of 16 hours prior to administration of the oral dose. This allows for more uniform absorption in all animals of the same species since the amount of food in the stomach varies greatly from animal to animal in the unfasted condition. Both rats and mice were weighed individually at the time of dosing to determine the proper injection volume. Glass syringes with special oral dosing needles were used to administer the compounds to the rats and mice.

Rangefinding doses were given for each compound. These consisted of dosing five rats and five mice at three levels determined from available evidence in the literature. After finding the proper range, geometrically spaced doses were administered to determine the LD_{50}. Ten male Sprague-Dawley albino rats and ten male CF-1 albino mice were dosed at each level and the LD_{50} with its 95% confidence limits were determined by the probit analysis method of Finney (1952). Deaths which occurred during the 14 days immediately following the administration of the single dose were included in the final mortality tally. Any animal that survived the 14-day postexposure observation period was sacrificed at that time.

The patch-test method was used to measure the degree of primary irritation of intact and abraded skin of female New Zealand albino rabbits. The rabbits were clipped of all possible hair on the backs and flanks 24 hours prior to exposure to allow for recovery of the skin from any abrasion resulting from the clipping. Six areas on the back, three on each side, were designated as patch areas. This allowed for the simultaneous testing of the three compounds on each rabbit, with each compound being tested on both the intact and abraded skin. Three areas on the right side of each rabbit were abraded by making minor incisions through the stratum corneum, but not sufficiently deep to disturb the derma or to produce bleeding. These were made in a square pattern with a syringe needle to make incisions. Six rabbits were tested for each compound.

The test material was applied in its native state in the amount of 0.5 gram for solids and 0.5 ml for liquids. The compound was applied to the designated areas and then covered by a one-inch square of surgical gauze, two single layers thick. The gauze patches were held in place with strips of elastoplast tape. The entire area was then covered with a rubber dental dam strip and secured with more elastoplast tape. The patches remained on the rabbits for 24 hours During that time, the rabbits were fitted with leather restraining collars to prevent disturbance of the patch area, but allowing the rabbits freedom of movement and access to food and water.

After 24 hours, the wrap and patches were carefully removed and the test areas evaluated for irritation using the Draize table as a reference standard. Readings were again made at 72 hours (48 hours after the first reading). The scores for each category in the table are the average score of the six rabbits tested. The higher the score, the more severe the irritation caused by the compound.

Skin absorption toxicity was determined using female New Zealand albino rabbits. All rabbits were clipped as closely as possible with an Oster clipper fitted with surgical blades. The back of the rabbits and the sides down to about half-way to the stomach area were clipped from the saddle area of the shoulders to the top of the rear leg area.

The rabbits were weighed prior to dosing to determine the proper dose volume. The compound was applied undiluted to the back of the rabbit, divided equally between the two sides of the rabbit. The compound was kept in place with 8-ply gauze patches. Latex rubber dental dam was then applied over the entire back area where clipped. Elastoplast tape was then used to keep the dose in place. Restraining harnesses, described earlier, were fitted to each rabbit during the entire dosing period.

All doses were kept in contact with the rabbits skin for 24 hours. After this period of time had elapsed, the tape, latex and gauze were removed and the animal was observed for 14 days. Three rabbits were dosed per level.

The inhalation exposures for 4-hour LC_{50} determinations were done using male, Sprague-Dawley, CFE rats and male CF-1 mice, ten per exposure level. All animals were observed for signs of toxicity and mortality during exposure and for the 14-days immediately following exposure. Animals were weighed immediately prior to exposure and again at 14 days postexposure on survivors. Determination of the LC_{50} was made by the probit method (Finney, 1952).

The three nitrotoluene isomers, having a low order of toxicity, were tested at saturated vapor concentrations. Production of essentially saturated vapors of the two liquid isomers was accomplished by bubbling dry air through a fritted disc immersed in the sample. The resultant vapors were then passed through a 9-liter glass chamber containing the experimental animals. The one solid isomer was tested by a static technique whereby an excess of the compound was sealed into a 120-liter plexiglass chamber for a period of 24 hours. The experimental animals were then rapidly introduced into the chamber by means of a sliding cage drawer designed to minimize vapor loss. All exposures were continuously analyzed using a Beckman Model 400 total hydrocarbon analyzer.

Analysis of tetranitromethane (TNM) was done using a chlorimetric method to determine contaminant concentration. In this method, Lyshkow reagent (modified Saltzman reagent) was allowed to mix and react with the sampled air in a glass delay coil. The resultant color developed was related to sample concentration and read using a Technicon AutoAnalyzer I system.

Standardization was based on a gravimetric technique using diffusion tubes. These were constructed by sealing the narrow end of disposable Pasteur pipettes resulting in straight wall tubes 110 mm long by 5 mm I.D., which when less than half full would diffuse approximately 3.5 µl of TNM vapor per minute. Two tubes showed a combined mean diffusion rate of 7.06 (s.d. $\pm$ 0.37) ppm/minute at 30 C.

For TNM generation, a modification of the diffusion tube system using two short tubes (30 mm length by 15 mm I.D.) gave a stable source of TNM from 70 µl/min with one tube at 30 C to 680 µl/min for two tubes at 60 C.

A Miran® infrared analyzer was used for the analysis of methyl nitrate concentrations. Calibration was achieved by injecting the proper volumes of liquid methyl nitrate into 30 liter standard bags to achieve concentrations in the 0-1000 ppm range. The conditions for the Miran® IR analyzer were as follows:

Pathlength	0.5 M
Slitwidth	1.0 mm
Wavelength	5.98
Gain	10 X
Absorbance	1.0 A
Time Constant	1.0
Sample Flow	500 cc/min.

A TNM ethanol solution was used for an intravenous LD_{50} determination in groups of rats and mice. Rats were injected using the lateral saphenous vein of the hind leg while mice were injected in the right lateral tail vein. The experimental animals were weighed prior to dosing to determine the proper injection volume. Ten animals were dosed at each level and the LD_{50} with its 95% confidence limits were determined by the probit analysis method previously mentioned.

Deaths that occurred during the 14 days immediately following the administration of the single dose were included in the final mortality. Any animal surviving the 14-day postexposure observation period was sacrificed at that time.

EXPERIMENTAL RESULTS & DISCUSSION

Tetranitromethane (TNM)

The rat 4-hour LC_{50} of TNM vapor was determined to be 17.5 ppm or 0.14 mg/liter. The mouse 4-hour LC_{50} for TNM is 54.4 ppm or 0.44 mg/liter (Table 1). Responses of the animals, within each species, were consistently dose-related and followed a general pattern of lethargy and inactivity with some eye and nose irritation at the toxic levels. All animals remained inactive during exposure with noticeable decrease in rate and depth of respiratory movements.

TABLE 1

4-HOUR INHALATION LC_{50} VALUES FOR RATS AND MICE EXPOSED TO TETRANITROMETHANE VAPOR (N = 10)

Rats		Mice	
Conc., ppm	Mortality Ratio	Conc.,ppm	Mortality Ratio
23	10/10	76	10/10
21	10/10	63	5/10
19	6/10	55	4/10
18	3/10	47	3/10
15	3/10	42	3/10
10	0/10	32	1/10
		17	0/10
		14	0/10
LC_{50} =	17.5 ppm	LC_{50} =	54.4 ppm
95% C.L. =	16.4 to 18.7 ppm	95% C.L. =	48.0 to 61.7 ppm

Deaths which occurred following exposure generally occurred within 12 hours. If the animals survived this time period, they usually lived to the 14-day sacrifice period. Rats at the non-lethal exposure level lost weight through the first four days postexposure but recovered and had normal weight gains by the end of the 14 days. Rats exposed at the partial mortality levels did not gain weight as fast and did not attain normal rates during the 14 days following exposure. Scattered weight losses were found in all mice which survived the 14 day observation period.

Gross pathological examination of the animals that died showed multifocal areas of moderate to severe lung congestion throughout all lobes with many of these areas appearing hemorrhagic. Animals examined after exposure to nonkilling levels of TNM vapors showed mild congestion of lungs upon gross examination.

Intravenous administration of TNM to rats and mice resulted in a LD_{50} of 12.6 mg/kg and 63.1 mg/kg, respectively (Table 2). As was seen in the results of inhalation exposures, rats are much more susceptible to TNM than mice. Mouse deaths occurred within minutes after dosing. Gasping with a foamy nasal discharge and tonic convulsions at the highest level preceded deaths in the rats.

Rat deaths occurred within two hours following administration of the compound. Control rats and mice given intravenous injections of equivalent volumes of ethanol alone had normal weight gains and survived the 14-day observation period.

TABLE 2

INTRAVENOUS TOXICITY OF TETRANITROMETHANE TO RATS AND MICE (N = 10)

Rats		Mice	
Dose, mg/kg*	Mortality Ratio	Dose, mg/kg*	Mortality Ratio
31.3	10/10	125	8/10
15.6	7/10	62.5	6/10
7.8	1/10	31.3	1/10

*Diluted in ethanol

LD_{50} = 12.6 mg/kg	LD_{50} = 63.1 mg/kg
95% C.L. = 10.0 to 15.9 mg/kg	95% C.L. = 45.0 to 88.7 mg/kg

Intragastric administration of undiluted tetranitromethane to fasted male rats produced an LD_{50} value of 130 mg/kg (Table 3). Similar administration of TNM to male mice produced a LD_{50} of 375 mg/kg. All animals remained inactive and lethargic for several hours after administration of the compound. Most deaths occurred during the 12-hour period immediately following dosing.

TABLE 3

ACUTE ORAL TOXICITY OF TETRANITROMETHANE IN RATS AND MICE (N = 10)

Rats		Mice	
Dose, mg/kg	Mortality Ratio	Dose, mg/kg	Mortality Ratio
500	10/10	1000	10/10
250	8/10	500	7/10
125	5/10	250	2/10

LD_{50} = 130 mg/kg	LD_{50} = 375 mg/kg
95% C.L. = 83 to 205 mg/kg	95% C.L. = 262 to 511 mg/kg

The difference between LD_{50} values determined for the oral and intravenous routes was most striking and suggestive of different mechanisms of toxicity dependent upon the route of administration. Additional studies were conducted to explore this unusual difference.

Groups of two rats each were given single oral doses of TNM and blood samples were taken 90 minutes after treatment. The blood samples were analyzed for methemoglobin content and a dose dependent response was measured as shown in Figure 1. In the rats dosed at 62.5 mg/kg and 125 mg/kg a second set of methemoglobin samples were collected three hours after exposure and the measured values had only decreased slightly below those found at 90 minutes as shown below:

Oral Dose mg/kg	Methemoglobin 90 Minutes g/100 ml	%	180 Minutes g/100 ml	%
62.5	3.5	28	3.3	26
125	5.9	47	5.3	42

The toxic signs observed in rats and mice after oral administration of TNM and the measured methemoglobin values are consistent with acute methemoglobinemia which is believed to be the toxic mechanism involved in lethality following this route of entry.

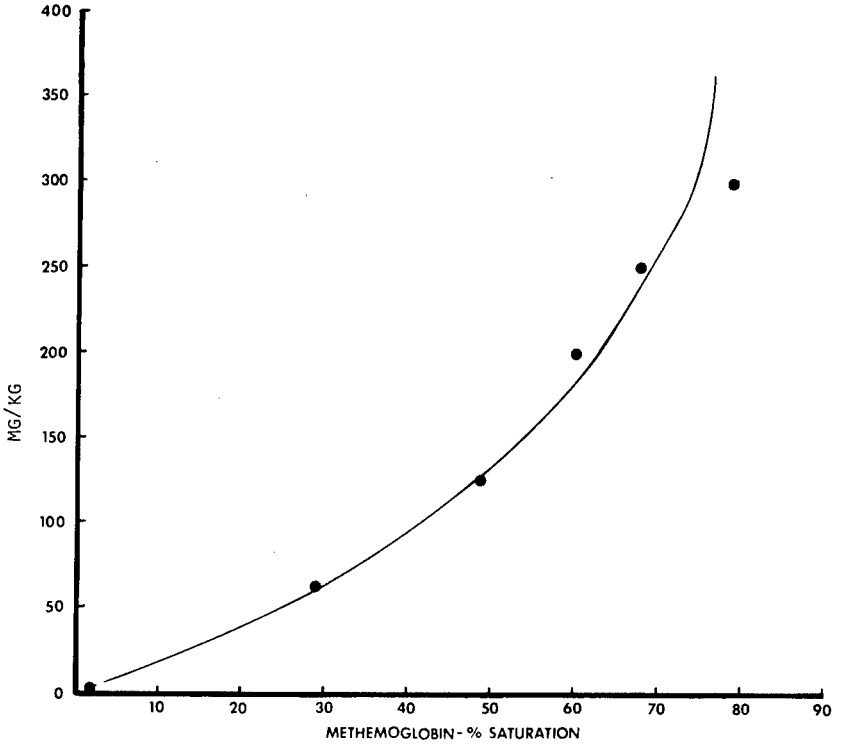

Figure 1. Methemoglobin response in rats given a single oral dose of tetranitromethane.

In order for TNM to form methemoglobin, the compound must be metabolized to nitrite ion. If NO_2 were liberated from the molecule it could be converted to nitrate and nitrate ions. The nitrate ion would then be converted to nitrite ion by bacterial action in the intestine. Since the mechanism of toxicity of ingested TNM appeared to be nitrite induced methemoglobinemia the rat oral LD_{50} was compared with the rat oral LD_{50} reported for sodium nitrate by Smyth et al. (1969) of 180 mg/kg. Calculation of the NO_2 content of each compound gives an adjusted LD_{50} for NO_2 of 120 mg/kg TNM and 122 mg/kg for sodium nitrite.

The signs of toxicity observed in the inhalation and intravenous exposures of rats and mice were consistent with acute pulmonary irritation and respiratory deaths. At necropsy animals exposed by these routes had congested and hemorrhagic lungs. Two rats were given an IV injection dose of 15 mg/kg TNM, slightly above the LD_{50} dose, and sampled for methemoglobinemia at 90 minutes and had the same methemoglobin values as untreated controls (0-3% saturation).

Animals exposed to TNM by the inhalation and intravenous routes reacted as if they had been exposed to nitrogen dioxide gas (NO_2). If one assumed that NO_2 was liberated from TNM in the lung on a equimolar basis (4 volumes of NO_2 for each volume of TNM) the measured TNM 4-hour LC_{50} value of 17.5 ppm would yield 70 ppm NO_2 which is comparable with the 4-hour LC_{50} for NO_2 of 88 ppm reported by Gray et al. (1954). Furthermore, a calculation of the dose of TNM inhaled by a 200 gram rat at the 4-hour LC_{50} concentration of 17.5 ppm assuming 100% absorption and a minute volume of 75 ml/min yields 12.6 mg/kg; a value identical to the IV LD_{50}.

Proof that TNM converts in the animal body to 4 equimolar volumes of NO_2 is difficult to obtain since methods for analysis of either compound are interchangeable and depend on the nitration of an aromatic ring with subsequent development of color than can be standardized. Physical chemical methods may be able to differentiate these compounds but lack sufficient sensitivity at the significant toxic concentrations. One possible exception may be electron spin resonance spectroscopy which should be capable of measuring NO_2 if released in physiological fluids because of its free radical character. We have not been able to explore this latter possibility since we have been unable to locate an ESR spectrometer.

Methyl Nitrate

The rat 4-hour LC_{50} of methyl nitrate vapor was experimentally determined to be 1275 ppm or 4 mg/liter and for mice the 4-hour LC_{50} for methyl nitrate is 5942 ppm or 18.7 mg/liter (Table 4). Responses of the animals were dose-related and followed a general pattern of lethargy, decreased respiratory rate and cyanosis. All animals were inactive throughout the 4-hour exposure period.

TABLE 4

4-HOUR INHALATION LC_{50} VALUES FOR RATS AND MICE EXPOSED TO METHYL NITRATE VAPOR (N = 10)

Conc.,ppm	Mortality Ratio	Conc.,ppm	Mortality Ratio
1608	10/10	7560	10/10
1285	5/10	6530	10/10
1121	1/10	6020	6/10
642	0/10	5800	3/10
		5500	0/10
		4990	0/10

LC_{50} = 1275 ppm

95% C.L. = 1200 to 1355

LC_{50} = 5942 ppm

95% C.L. = 5827 to 6509 ppm

Rats that died as a result of vapor exposure died either during exposure or in the following 12 hours. Mouse deaths were often delayed, ranging from 3 to 11 days postexposure. With few exceptions, rats and mice that survived the 14-day postexposure period showed normal body weight gains.

Gross pathological examination of the rats and mice that died showed mild to moderate pulmonary congestion with focal areas of hemorrhage. Nonlethal levels provided similar gross pathologic results.

Single peroral doses of methyl nitrate to fasted male rats produced an LD_{50} value of 344 mg/kg as shown in Table 5. Administration of methyl nitrate to fasted male mice by the peroral route resulted in an LD_{50} of 1820 mg/kg. Both rats and mice were inactive and lethargic immediately following the intragastric dosing. Rats also exhibited labored breathing and gasping at the highest dose level. Deaths were seldom delayed with most occurring during the 12-hour period immediately following dosing.

The toxicity of methyl nitrate is much greater in rats than in mice by either oral or inhalation routes of exposure. This difference of approximately 5-fold may be a function of body size and if so it could be significantly more toxic in man. Additional acute studies should be conducted with larger species to determine if the toxicity of methyl nitrate is size dependent.

TABLE 5

ACUTE ORAL TOXICITY OF METHYL NITRATE TO RATS AND MICE (N = 10)

Rats		Mice	
Dose,mg/kg	Mortality Ratio	Dose,mg/kg	Mortality Ratio
1000	10/10	2000	8/10
500	10/10	1800	6/10
397	6/10	1590	0/10
315	4/10	1260	0/10
250	1/10		
LD_{50} =	344 mg/kg	LD_{50} =	1820 mg/kg
95% C.L. =	308 to 384 mg/kg	95% C.L. =	1738 to 1906 mg/kg

Nitrotoluenes

Exposures to essentially saturated vapors of each of the nitrotoluene isomers, ortho, meta and para, resulted in no deaths of either rodent species. The saturation concentrations at 22 C (chamber temperature) of the nitrotoluenes were calculated from the Antoine Equation (Lange, 1956).

$$\text{Log } P = \frac{-52.23B}{T} + C_1$$

where T is temperature in Kelvin and B and C are the constants below.

Isomer	B	C	Temperature Limits (C)
ortho	48.114	7.9728	50-225
meta	50.128	8.0655	55-235
para	49.95	7.9815	80-240

Although the lower temperature limits of this equation are higher than the exposure chamber temperature, the values were used to check saturation concentrations obtained in standard bags. The following table compares the experimental and theoretical values.

Compound	Saturation Concentration, ppm Theoretical*	Experimental
ortho	392	416
meta	200	203
para	174	228

*Obtained from Antoine Equation.

The theoretical and experimental values for the ortho and meta isomers are in good agreement. The greater deviation of the theoretical value for para-nitrotoluene from experimental is probably due to the fact that the lower temperature limit of the Antoine Equation for this compound is 80 C, much higher than for the other two. Extrapolation to chamber temperature might, therefore, lead to greater error for para-nitrotoluene.

Rats and mice were exposed for 4-hours to the highest concentrations attainable for these compounds. The measured concentrations obtained are listed below.

Compound	Species	Conc.,ppm	Percent of Saturation
ortho	Rats	320	77
	Mice	354	85
meta	Rats	157	77
	Mice	151	74
para	Rats	152	67
	Mice	228	100

No deaths occurred in any of the exposures or in the subsequent 14-day observation period. All animals gained weight normally during the 14-day observation period. Gross pathological examination of animals sacrificed after 14 days revealed no lesions which could be attributed to exposure. The experimental results show no observable adverse effects on rodents from 4-hour exposures to essentially saturated vapors of the nitrotoluenes.

When held in covered contact with the clipped trunks of female albino rabbits for 24 hours, the undiluted nitrotoluene isomers, ortho, meta and para, produced no observable toxic effects. The dose level of 20 g/kg was not absorbed during the 24-hour period and increasing the dose beyond this level would be uninformative. All rabbits were symptom free and gained weight normally during the subsequent 14-day observation period.

Primary irritation tests on intact and abraded skin were negative for all three nitrotoluene isomers. Readings taken at 24 and 72 hours averaged at less than one per rabbit using the Draize method of scoring. These scores indicate a complete lack of skin irritating potential for these three compounds.

The isomers of nitrotoluene, ortho, meta and para, were essentially nontoxic by the inhalation and transdermal routes of administration. None of the isomers was irritating to the skin of rabbits after 24 hours. These findings are not surprising since the end products of the metabolism of p-nitrotoluene in the dog are reported by Williams (1959) to be p-nitrobenzoic acid and p-nitrohippuric acid. Williams further states that the primary products of o-nitrotoluene are o-nitrobenzoic acid and a glucuronide of o-nitrobenzyl alcohol. All of the metabolites are readily excreted and are considered essentially nontoxic. The metabolic fate of m-nitrotoluene has not been identified but it probably follows similar pathways since its low order of toxicity is comparable to the other isomeric forms.

CONCLUSIONS

TNM exhibits 2 types of acute toxic responses depending on the mode of administration. The toxic effects after ingestion of TNM are characteristic of acute methemoglobinemia and are qualitatively and quantitatively equivalent to those caused by nitrite intoxication under the premise that all NO_2 groups in TNM are converted to nitrite. Animals acutely exposed to TNM by the inhalation and intravenous routes experience severe pulmonary irritation and no methemoglobinemia. Results of 4 hour inhalation exposures indicate that the effects of TNM are similar to those obtained from NO_2 gas at a molar concentration 4 times that of the TNM. It is planned that investigations into the comparative toxicity of TNM and NO_2 under subchronic conditions will be made to resolve the question of toxic equivalency of the 2 materials. In contrast to TNM, the oral and inhalation toxicities of methyl nitrate are quite comparable when the LC_{50} is converted to a dose. Using the same assumptions of animal size and minute respiration volume, the LC_{50} for rats converts to 360 mg/kg almost identical to the oral LD_{50} of 344 mg/kg. Because methyl nitrate is much more toxic to rats than mice, the oral LD_{50} of this material in guinea pigs will be measured. Since this species is larger than rats, results of acute studies should demonstrate whether or not an inverse relationship exists between toxicity of methyl nitrate and body size.

No toxic effects were observed after rabbit skin exposure to 20 g/kg or rat and mouse inhalation exposures to essentially saturated vapors of the three isomeric nitrotoluenes. This information with application of a safety factor may be sufficient for establishment of emergency exposure limits for these materials.

SECTION II

COMPARATIVE SUBCHRONIC TOXICITY OF TETRANITROMETHANE AND NITROGEN DIOXIDE: ACUTE TOXICITY OF METHYL NITRATE TO GUINEA PIGS

E. H. Vernot, J. D. MacEwen, E. R. Kinkead and C. C. Haun

INTRODUCTION

For many years, exposure to tetranitromethane (TNM) has been a hazard associated with the manufacture and processing of trinitrotoluene (TNT) Because of this, some early animal studies were carried out in efforts to characterize the toxicity of TNM. Sievers et al. (1947) performed inhalation exposures of cats using impure TNM either in TNT or isolated from the TNT manufacturing process. They found that concentrations of 0.1 - 0.4 ppm were mildly irritating to the eyes during 6 hour exposures. Concentrations of 3-7 ppm caused severe irritation of the eyes, mouth and upper respiratory tract and also resulted in pulmonary edema. Exposure to 7 ppm or over killed all cats within 6 hours. Horn (1954) exposed 20 rats to 33 ppm TNM which killed all animals in 10 hours. A 6-month exposure to 6.35 ppm led to the deaths of 11 of 19 rats although 2 dogs exposed similarly showed only mild toxic signs. Korbakova (1960) determined the 2-hour LC_{50} to mice to be 75 ppm and noted effects on the conditioned reflex activity of rats after 1 hour exposures to 0.4 ppm.

Since TNM is a byproduct of the manufacture of TNT and has the capability of escaping into the atmosphere and affecting areas surrounding munition plants, the US Army has a strong interest in determining its toxicity characteristics. Previous work in this laboratory had measured the acute toxicity of TNM by various routes of administration to give the values shown in Table 6.

TABLE 6

ACUTE TOXICITY OF TNM TO RATS AND MICE

	Rats	Mice
Oral LD_{50} (95% C.L.)	130 (83-205) mg/kg	375 (262-511) mg/kg
Intravenous LD_{50} (95% C.L.)	12.6 (10.0-15.9) mg/kg	63.1 (45.0-88.7) mg/kg
4-Hour Inhalation LC_{50} (95% C.L.)	17.5 (16.4-18.7) ppm	54.4 (48.0-61.7) ppm

Intravenous and inhalation toxicity appeared to be almost identical since the total rat dose achieved by the 4-hour inhalation was the same as the rat intravenous LD_{50}. In both cases the cause of death appeared to be severe lung damage. Additionally, the inhalation toxicity to rats of TNM was quantitatively 5 times greater on a molar basis than that of nitrogen dioxide (NO_2) reported by Gray et al., (1954) to be 88 ppm. Since the lethality of NO_2 is also due to its lung irritant properties leading to lung edema and hemorrhage, it seemed reasonable to hypothesize the toxicity of TNM was due to the action of the NO_2 moieties in the molecule leading to an LC_{50} approximately 1/4 that of NO_2.

In order to determine whether the relationship between the toxicities of NO_2 and TNM held constant at subacute levels, this additional study was planned with the rationale that a constant ratio at acute and subacute levels would imply a like relationship for chronic exposure. Exposure levels, both industrial and public, could then be set for TNM on the basis of presently accepted values for NO_2.

Acute studies with methyl nitrate had provided oral LD_{50} values for rats of 344 mg/kg and for mice of 1820 ppm, while the 4-hour inhalation LC_{50} for rats was 1275 ppm and that for mice was 5942. This 5-fold greater toxicity to rats was probably due to differences in metabolic transformation in the two species. However, the possibility existed that the toxicity difference was inversely related to animal size or body surface area. In order to have a basis for extrapolation of the results to man, a much larger organism, the latter possibility had to be investigated. Therefore, this study included a determination of the acute oral toxicity of methyl nitrate to guinea pigs. The guinea pig is small enough to permit use of enough animals to attain statistical validity, and yet sufficiently large, 2-3 times the size of the rats used in the study, to reveal an effect of body size if one exists.

MATERIALS AND METHODS

TNM and NO_2

Simultaneous, 2-week continuous exposures were conducted on equivalent concentrations of TNM and NO_2 assuming the NO_2 groups in TNM to be acting independently. NO_2 concentrations were 4 times those of TNM except for the last exposure which was over 5 times the TNM concentration. Exposure groups consisted of 100 male rats, housed 10 per cage in Longley Chambers (MacEwen, 1965). Two smaller Rochester Chambers (Leach et al., 1959) housed the control rats which were exposed to air alone.

The concentrations used in the experiments were as follows:

Experiment No.	TNM Conc., ppm	NO_2 Conc., ppm
1	7.5	30
2	5.0	20
3	3.5	14
4	7.5	40

Several problems occurred during the third and fourth day of the first exposure to 7.5 ppm TNM which resulted in concentration excursions. Two excursions, although for short duration, exceeded the 4-hour LC_{50} of 17 ppm. The following day four TNM rats died. Although the problems were eliminated and concentration control was satisfactory thereafter, the second exposure to 7.5 ppm TNM (Experiment No. 4) was planned because of the uncertainty caused by the excursions. Since the 30 ppm NO_2 had proceeded satisfactorily, an exposure to 40 ppm NO_2 was included in Experiment No. 4.

All rats were examined daily for general appearance, behavior, signs of toxic stress and lethality with body weights recorded immediately prior to the start of exposure and at the conclusion, 14 days later.

Twenty rats per group including controls were reserved for methemoglobin determinations (MacEwen and Vernot, 1970) at the conclusion of the 14 day exposure period. In addition, groups of rats (consisting of 10 per group from the first experiment and 20 per group for all other experiments) had lungs precisely removed for wet weight determinations (Vernot and Kinkead, 1975). The wet lung weights of each group were statistically analyzed for determination of edematous effects. The remaining animals were sacrificed and livers and kidneys weighed during necropsy.

Gross and histopathologic examinations were made on all animals that died during exposure or were sacrificed at the conclusion of the 14-day study. Organ weights of lung, liver and kidneys were recorded for all animals at sacrifice. Statistical comparisons (Student's t test) were performed on the mean organ weights.

The contaminant concentrations were continuously monitored using a colorimetric method in which a modified Saltzman reagent was allowed to mix and react with the sampled air in a glass delay coil. The resultant color developed was then related to the sample concentration and read using a Technicon AutoAnalyzer system.

In addition, a Wilkes Miran IR® infrared analyzer was also used to monitor the TNM chamber at a wavelength of 7.84 μm. The absorbance measurement of the Miran was continuously recorded on a strip chart recorder. The purpose of dual analysis was to determine if any dissociation of tetranitromethane into nitrogen dioxide took place in the exposure chamber which would result in a decrease in infrared absorbance without affecting the Auto-Analyzer result, since the AutoAnalyzer measures NO_2 as efficiently as TNM. There proved to be no evidence of spontaneous dissociation during the two days; therefore, use of the AutoAnalyzer system for monitoring TNM concentrations was discontinued.

Analytical instrument calibration was based on a gravimetric technique using diffusion tubes. For TNM, these were constructed by sealing the narrow end of disposable Pasteur pipettes resulting in straight wall tubes 110 mm long by 5 mm I.D., which when less than half full would diffuse approximately 3.5 μl of TNM vapor per minute in an AID® Model 303 oven. Two tubes showed a combined mean diffusion rate of 7.06 (s.d. ± 0.37) per/minute at 30 C. For NO_2, commercially available Teflon® permeation tubes were used for calibration.

For TNM generation, a modification of the diffusion tube system using two short tubes (30 mm length by 15 mm I.D.) gave a stable source of TNM from 70 μl/minute with one tube at 30 C to 680 μl/minute for two tubes at 60 C. The introduction system for NO_2 consisted of a flask containing liquid NO_2 in a thermostatted cold water bath. The NO_2 was vaporized into a stream of carrier air and delivered into the exposure chamber. Concentration was controlled by adjusting bath temperature (0-10 C), carrier gas flow and chamber air flow.

Electron paramagnetic resonance (EPR) spectra of whole blood to which TNM had been added were obtained in an effort to discover free radical species, possibly NO_2, produced. The instrument used was a Varian Associates Fieldial Mark I EPR spectrometer. After preliminary experiments, the following conditions were developed for in vitro blood examination:

1. Cool 16 ml whole blood or plasma to 0 C.
2. Mix 160 mg TNM with blood or plasma at 0 C.
3. Freeze mix to -170 C for EPR scan.

These were the only conditions resulting in EPR spectra; no signals were obtained when scans were run at 0 C or room temperature on either controls or reactants.

Methyl Nitrate

Glass syringes with special oral dosing needles were used to administer pure methyl nitrate to guinea pigs. The experimental animals were fasted for at least 16 hours prior to

administration of the oral dose. This allowed for uniform absorption in all animals since the amount of food in the stomach varies greatly from animal to animal in the unfasted condition. Guinea pigs were weighed individually at the time of dosing to determine the proper injection volume.

Ten guinea pigs were used at each oral dose level and the LD_{50} calculated using the probit analysis method of Finney (1952). Deaths which occurred during the 14 days immediately following the administration of the single oral dose were included in the final mortality. All that survived the 14-day postexposure observation period were sacrificed at that time.

Gross pathologic examinations were performed on all guinea pigs that died following the administration of the oral dose and on representative animals from groups sacrificed at the conclusion of the 14-day holding period.

EXPERIMENTAL RESULTS

TNM and NO_2

Rats exposed to the highest levels of both contaminants showed lethargy, dyspnea, kyphosis and general poor health, but TNM exposed animals exhibited these symptoms of toxic stress to a greater degree than those exposed to comparable NO_2 concentrations. In addition, TNM caused a noticeable yellowing of the fur. Although the toxic signs decreased with decrease in concentration, they were still visible at the lowest concentrations tested.

The methemoglobin concentrations found in the blood of rats after 2-weeks exposure to various concentrations of TNM and NO_2 are listed in Table 7 along with their respective controls. It is obvious that inhalation of either toxicant does not cause significant methemoglobinemia.

TABLE 7

METHEMOGLOBIN IN BLOOD OF RATS EXPOSED TO TNM OR NO_2 FOR TWO WEEKS

Contaminant	% Methemoglobin[1]	Contaminant	% Methemoglobin
Control	0.76 ± 0.11[2]	Control	1.01 ± 0.13
3.5 ppm TNM	0.92 ± 0.15	7.5 ppm TNM[3]	1.23 ± 0.31
14 ppm NO_2	0.99 ± 0.16	30 ppm NO_2	1.25 ± 0.27
Control	0.95 ± 0.12	Control	1.26 ± 0.22
5.0 ppm TNM	1.00 ± 0.16	7.5 ppm TNM	1.08 ± 0.13
20 ppm NO_2	1.21 ± 0.16	40 ppm NO_2	1.30 ± 0.26

[1]% of total hemoglobin
[2]95% confidence limits.
[3]Exposure which experienced concentration excursions.

The cumulative mortality data for rats exposed to various concentrations of TNM are detailed in Table 8. The effect of the concentration excursions on the third and fourth day of the first exposure at 7.5 ppm TNM which twice exceeded 17 ppm for short periods, is seen as an increased mortality during the period from 5 to 9 days. Thereafter, the mortality in the initial and repeat 7.5 ppm studies were very similar. In Figure 2 separate comparisons are made between the mortality curves resulting from the second 7.5 ppm exposure and those from 30 and 40 ppm NO_2. There appear to be no similarities between either of the NO_2 curves and that of TNM. In both Figure 2A and 2B the 7.5 ppm TNM curve intersects the NO_2 curves. In Figure 2B, mortality in the 40 ppm exposure increased rapidly until the third day, then leveled off until the tenth day when an increase began again.

TABLE 8

CUMULATIVE MORTALITY OF RATS CONTINUOUSLY EXPOSED TO TNM OR NO_2*

(% Mortality)

Days of Exposure	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Compound and Conc., ppm						(Experiment 1)								
TNM - 7.5	0	0	1	1	5	8	12	17	21	24	31	44	58	75
NO_2 - 30.0	0	3	3	3	3	5	7	7	9	9	14	18	21	31
						(Experiment 2)								
TNM - 5.0	0	0	0	0	0	0	0	2	2	5	6	9	11	16
NO_2 - 20.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
						(Experiment 3)								
TNM - 3.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NO_2 - 14.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
						(Experiment 4)								
TNM - 7.5	0	0	1	1	1	1	3	7	14	23	30	42	54	65
NO_2 - 40	0	21	26	27	27	27	27	27	28	29	35	41	46	50

*Number of animals initially in each exposed group = 100.

Tables 9 through 12 detail mean body and organ weights obtained at termination of the exposure periods. Although there are large decreases in control and exposed liver and kidney weights when compared to controls, these do not seem to be related to dose in any regular fashion, e.g. liver weights of rats exposed to 5 ppm TNM are higher relative to controls than are those for rats exposed to 3.5 ppm and liver weights of animals exposed to 20.0 ppm NO_2 are larger than controls. It is obvious however, that there are dose related decreases in body weight and increases in lung weight at the termination of exposure.

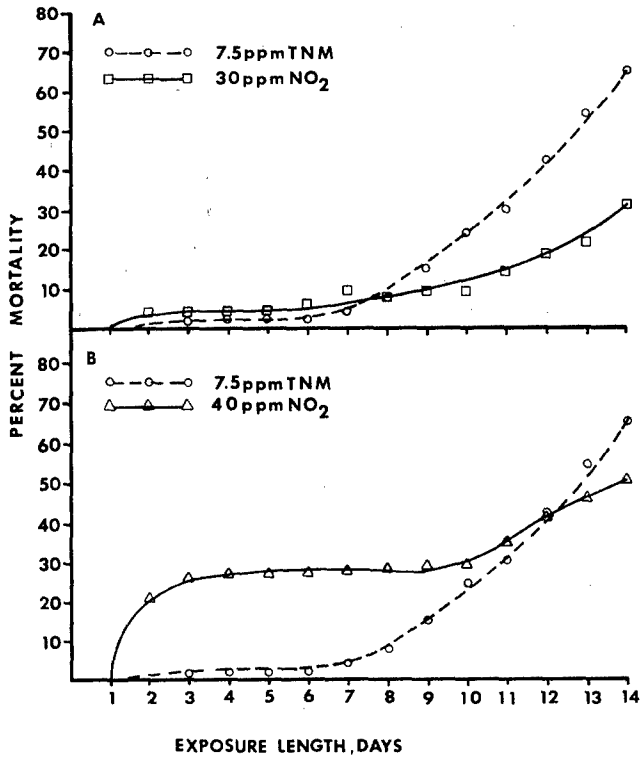

Figure 2. Comparison of mortality during exposure to TNM and NO_2.

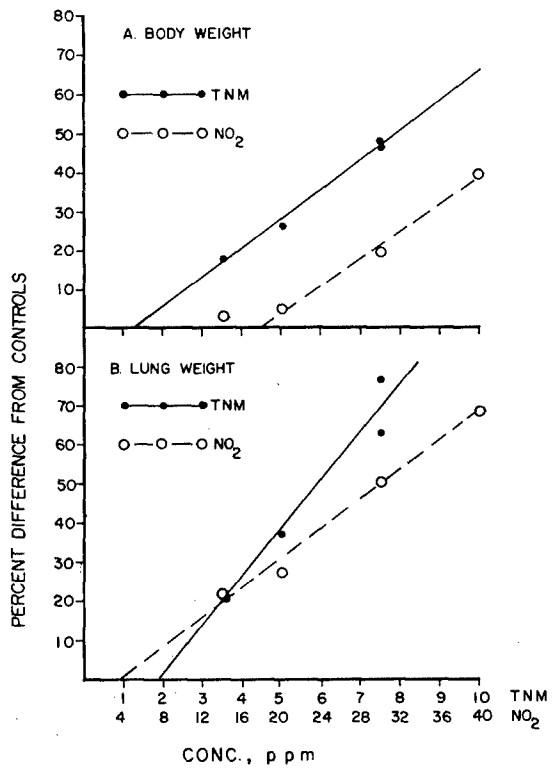

Figure 3. Dose related effects of exposure to TNM and NO_2.

TABLE 9

SUMMARY OF EFFECTS OF 14-DAY CONTINUOUS EXPOSURE TO 3.5 PPM TNM OR 14 PPM NO_2 ON RATS

	Controls	TNM	NO_2
$\bar{x}$ Body Weight at Start, g (N = 100)	247.3	246.2	243.4

Summary of Wet Lung Examination, N = 20

	Controls	TNM	NO_2
$\bar{x}$ Body Weight, g	319.0	264.6[1,2]	310.4
$\bar{x}$ Lung Weight, g	1.511	1.821[1]	1.841[1]

Summary of Body and Organ Weights at Termination

	Controls, N = 60	TNM, N = 60	NO_2, N = 60
$\bar{x}$ Body Weight, g	319.7	258.7[1,2]	305.4[1]
$\bar{x}$ Liver Weight, g	11.982	9.217[1,2]	10.000[1]
$\bar{x}$ Kidney Weight, g	2.680	2.358[1,2]	2.569[1]

[1]Different from controls at the 0.01 level of significance.

[2]Different from NO_2 group at the 0.01 level of significance.

TABLE 10

SUMMARY OF EFFECTS OF 14-DAY CONTINUOUS EXPOSURE TO 5 PPM TNM OR 20 PPM NO_2 ON RATS

	Controls	TNM	NO_2
$\bar{x}$ Body Weight at Start, g (N = 100)	199.0	199.7	199.1

Summary of Wet Lung Examinations, N = 20

	Controls	TNM	NO_2
$\bar{x}$ Body Weight, g	262.3	194.5[1,2]	250.1
$\bar{x}$ Lung Weight, g	1.401	1.922[1,3]	1.784[1]

Summary of Body to Organ Weights at Termination[4]

	Controls, N = 60	TNM, N = 49	NO_2 N = 60
$\bar{x}$ Body Weight, g	263.4	198.3[1,2]	254.9[1]
$\bar{x}$ Liver Weight, g	8.405	6.833[1,2]	9.063[1]
$\bar{x}$ Kidney Weight, g	2.012	1.551[1,2]	1.995

[1]Different from controls at the 0.01 level of significance.

[2]Different from NO_2 group at the 0.01 level of significance.

[3]Different from NO_2 group at the 0.05 level of significance.

[4]Mortality in TNM group precluded sampling 60 rats at termination.

TABLE 11

SUMMARY OF EFFECTS OF 14-DAY CONTINUOUS EXPOSURE TO 7.5 PPM TNM OR 30 PPM NO_2 ON RATS

	Controls	TNM	NO_2
$\bar{x}$ Body Weight at Start, g (N = 100)	187.8	184.5	186.8

Summary of Wet Lung Examination, N = 10

	Controls	TNM	NO_2
$\bar{x}$ Body Weight, g	267.8	143.1[1,2]	215.6[1]
$\bar{x}$ Lung Weight, g	1.290	2.274[1,3]	1.933[1]

Summary of Body and Organ Weights at Termination[4]

	Controls, N = 69	NO_2, N = 48
$\bar{x}$ Body Weight, g	275.7	209.8[1]
$\bar{x}$ Liver Weight, g	10.987	7.683[1]
$\bar{x}$ Kidney Weight, g	2.371	1.935[1]

[1]Different from controls at the 0.01 level of significance.

[2]Different from NO_2 group at the 0.01 level of significance.

[3]Different from NO_2 group at the 0.05 level of significance.

[4]No TNM exposed animals available for sampling at termination. Mortality in NO_2 group precluded sampling 60 rats at termination. Extra control animals available for sampling at termination.

TABLE 12

SUMMARY OF EFFECTS OF 14-DAY CONTINUOUS EXPOSURE TO 7.5 PPM TNM OR 40 PPM NO_2 ON RATS

	Controls	TNM	NO_2
$\bar{x}$ Body Weight at Start, g (N = 100)	254.7	254.4	256.4

Summary of Wet Lung Examinations, N = 20

	Controls	TNM	NO_2
$\bar{x}$ Body Weight, g	327.6	171.9[1,2]	199.1[1]
$\bar{x}$ Lung Weight, g	1.651	2.687[1]	2.780[1]

Summary of Body and Organ Weights at Termination[3]

	Controls, N = 60	NO_2, N = 48
$\bar{x}$ Body Weight, g	315.4	210.5[1]
$\bar{x}$ Liver Weight, g	10.745	6.789[1]
$\bar{x}$ Kidney Weight, g	2.573	1.874[1]

[1]Different from controls at the 0.01 level of significance.

[2]Different from NO_2 group at the 0.01 level of significance.

[3]No TNM exposed animals available for sampling at termination. Mortality in NO_2 group precluded sampling 60 rats at termination.

When concentration is plotted against the percent difference of test animals from their respective controls in body weight as in Figure 3A or lung weight as in Figure 3B, linear relationships are revealed for both NO_2 and TNM. The plots in Figure 3 are regression lines with the following correlation coefficients: TNM lung weight - 0.974, NO_2 lung weight - 0.994, TNM body weight - 0.996, NO_2 body weight - 0.996. The body weight difference at 14 ppm NO_2 was judged to be insignificant and was not used in calculating the regression line. Concentration units on the abscissa are set up so that NO_2 concentrations are 4 times those of TNM.

The EPR spectra of plasma showed no change when TNM was added. However, when TNM was mixed with whole blood, strong absorption peaks appeared at $g = 2$ and $g = 6$ as shown in Figure 4. The factor g is a complex ratio of microwave frequency to magnetic field strength. These spectra are similar to those described by Peisach et al. (1968) produced by reaction of ferricyanide with hemoglobin where the heme is converted to a high spin ferric form or, in other words, to methemoglobin. The production of methemoglobin was confirmed by spectrophotometry in the visible region.

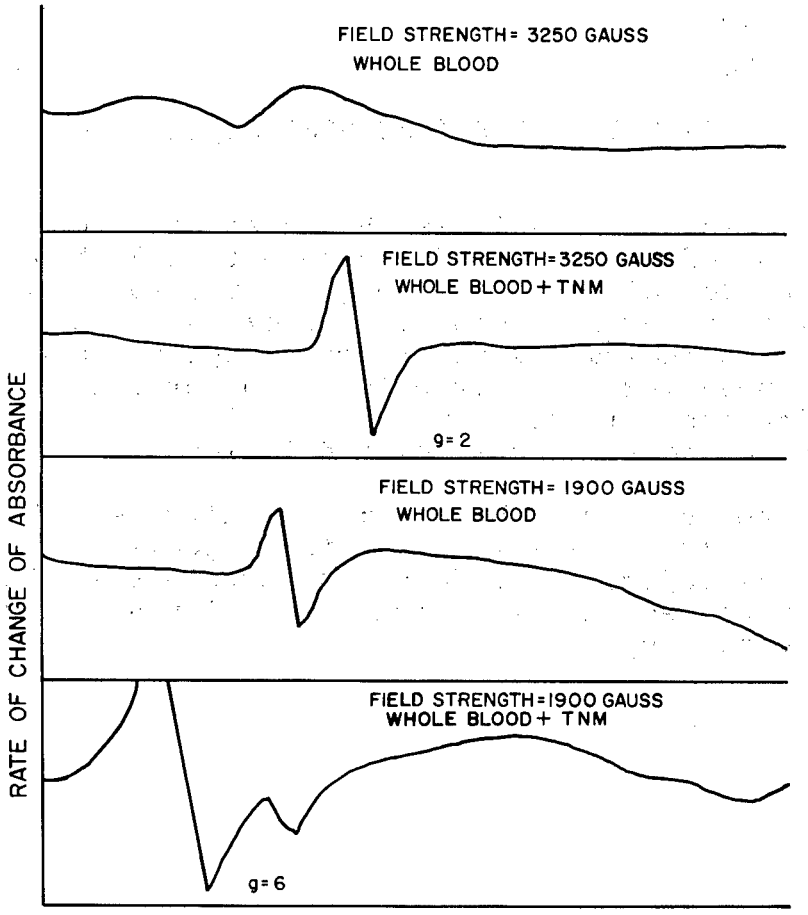

Figure 4. EPR spectra of whole blood with and without TNM.

As was detailed in the Methods Section, it was necessary to cool blood to 0 C and freeze it to -170 C to produce EPR. No evidence of methemoglobin was obtained when mixing took place at room temperature, indicating that methemoglobin formation would not be expected at body temperature.

Methyl Nitrate

Table 13 summarizes the oral toxicity information obtained with guinea pigs.

TABLE 13

ACUTE ORAL TOXICITY OF METHYL NITRATE TO GUINEA PIGS

Dose Level, mg/kg	Mortality Ratio
1000	10/10
800	7/10
600	7/10
500	3/10
400	1/10
200	1/10

LD_{50} and 95% C.L. = 548 (456-658) mg/kg.

All but two of the animals that died as a result of peroral administration of the compound did so within 24 hours of dosing. Gross examination of the guinea pigs that died following the single oral dose revealed chocolate-brown discoloration of the blood and lungs indicating severe methemoglobinemia. Except for the livers appearing slightly pale, no other lesions were observed. Gross examinations of the animals surviving the 14-day observation period revealed no treatment related lesions.

The guinea pig oral LD_{50} of methyl nitrate, 548 mg/kg, demonstrates that it is less toxic to this species than to rats with an LD_{50} of 344 mg/kg. Therefore, the hypothesis of an inverse relationship between methyl nitrate toxicity and body surface area or size was rejected.

DISCUSSION

Comparison of the mortality curves in Figure 2 suggests that there is a difference in the mode of action of NO_2 and TNM. Although at 14 days the mortality resulting from exposure to 7.5 ppm TNM is much higher than that from 30 ppm NO_2, there is an indication of higher mortality early during exposure to NO_2. This is seen much more clearly in the 40 ppm NO_2 exposure where the course of deaths is biphasic with 21 deaths after 2 days and a phase between the 4th and 9th days where no deaths occurred. In contrast, the mortality curve resulting from TNM exposure is smoothly rising.

Because the concentration scales of Figure 3 are drawn so that NO_2 units are 4 times larger than TNM units, one would expect superposition of the lines in 2A and 2B if the effects of NO_2 and TNM were the same on a 4 to 1 concentration basis. Obviously, this is not true for either parameter, body weight or lung weight. Although the effects of the toxicants on rat body weight appear to be similar in that the dose response lines are parallel, this does not provide a constant potency ratio, and the potency of TNM is always greater than 4 times that of NO_2. No simple comparison can be made for lung weight effects where the dose response lines are not parallel. Above 3.5 ppm, TNM lung effects are relatively greater than 4 to 1 compared to NO_2, and below 3.5 ppm they are relatively less. Extrapolation of the dose response lines to zero effect provides a concentration of 3.5 ppm for NO_2 and 1.9 ppm for TNM a ratio of 1.8 to 1.

Using the parameters of mortality, lung weight increase and body weight gain rate decrease as indices of comparison, it is seen that the initial hypothesis of 1 to 4 molar equivalency of toxicity of TNM to NO_2 is not confirmed by the experimental facts. Quantitatively, the ratio is different from 1:4 in all parameters tested, lung weight, body weight and mortality. There appear to be qualitative differences as well as shown by the biphasic character of the 40 ppm NO_2 mortality curve and the appearance of the lung weight plot.

Examination of the appendix to this report detailing the histopathological lesions induced by exposure to various concentrations of TNM and NO_2 indicates that the incidence of lung edema is the best gauge of the toxic action of TNM and NO_2. No edemagenic effect occurs upon exposure to 14 and 20 ppm NO_2 for two weeks while there is a concentration related incidence of edema in all exposures to TNM. Edema formation is the same in TNM and NO_2 exposed rats only at the 7.5 ppm TNM and 40 ppm NO_2 levels, a concentration ratio of 5.3 NO_2/1 TNM.

CONCLUSIONS

The actions of TNM and NO_2 during 2-week inhalation exposures are similar in the sense that the only effects appear to be those related to lung irritation and edemagenesis with no primary lesions in any other organ.

Results of this series of 2-week exposures demonstrate that the two compounds are not toxicologically equivalent on a 4 mole NO_2 to 1 mole TNM basis as postulated from acute toxicity test results.

Data developed in this study can be used to establish emergency exposure limits for TNM since concentrations having minimal effects after two weeks exposure would not represent hazards for short term exposure.

Because this study has demonstrated the toxicological nonequivalency of TNM and NO_2, it can not be used for the recommendation of chronic exposure limits (TLV's) for TNM. Only long-term animal studies using this contaminant would be satisfactory for such recommendation.

The fact that the acute oral toxicity of methyl nitrate to guinea pigs is less than that to rats demonstrates that there is no inverse relationship between body size and toxicity. Extrapolation to man can then be made without consideration of this possibility.

APPENDIX

Pathological Changes seen in the Tissues of Rats Exposed to Tetranitromethane (TNM) and Nitrogen Dioxide (NO_2) by the Inhalatory Route

A. General Discussion

Histopathologic lesions seen were of three types: (1) Changes in the respiratory system that were related to exposure sometimes, but not always, in a dose-dependent way. These changes were, in the main, attributable to irritation of epithelium in the tracheobronchial tree and lung alveoli. The primary changes due to irritation are largely exudative, but some proliferative lesions were seen. Lung edema was deemed the most severe primary lesion seen and its incidence was closely correlated with mortality. Secondary disease occurred within the lungs. Secondary disease was predominantly pneumonia and structural changes in lung architecture, and these lesions were associated with primary profound exudation of either mucus and/or edema fluid in the tracheobronchial tree or lung alveoli. (2) Lesions seen in other organs (liver, heart, kidneys) were associated with death of the animal or altered hemodynamics due to advanced disease in the lungs. These lesions were predominantly distention of blood filled vasculature. The presence of these lesions and their degree of severity were positively correlated with fatal lung disease or with advanced lung disease at necropsy. (3) Incidental findings occurred sporadically within all systems examined, but could not be related to the exposure. These incidental lesions were compatible with those routinely seen in experimental rats or those seen as agonal phenomena.

B. Respiratory Changes in Rats Exposed to TNM (see Table 14)

A form of pneumonitis characterized by interstitial proliferation and/or accumulation of histiocytes, plasma cells, lymphocytes and fibroblasts with multifocal distribution associated with the distal ends of terminal bronchioles was seen in a high percentage of rats exposed to all concentrations of TNM (78% of exposed rats vs 27% of control rats). Neither incidence nor degree of severity of pneumonitis were concentration dependent. This lesion is interpreted as a sensitive and mild nonspecific response by the rat lung to a pulmonary irritant. It should be noted in passing that the excessive incidence of this lesion in control rats for the highest level contaminant exposures was due to a high incidence of early and mild lesions of chronic murine pneumonia seen in these control animals. Catarrhal bronchitis and/or bronchiolitis was seen in a high percentage of rats exposed to all three concentrations of TNM. This lesion was characterized by accumulations of mucus in the lumina of the middle size and small airways. There

was a 74% incidence of this lesion in all exposed rats vs 4% in control rats. While the incidence of this lesion was not concentration dependent, the degree of severity was elevated in the lungs of rats exposed to the high dose of TNM (7.5 ppm). Here again, this lesion is interpreted as a sensitive response by the bronchial tree to a pulmonary irritant. The number of mucus-laden goblet cells seen in the bronchial epithelium correlates well with the amounts of mucus seen in the lumen. In addition, there was an increase in the volume of the cytoplasm of bronchial epithelium; and this cytoplasm was pale and finely vacuolated. This change was most noticeable in the 5.0 and 7.5 ppm dose groups. Cilia on the surface of bronchial epithelium were organized and often appeared stubby in rats exposed to 5.0 and 7.5 ppm of TNM. Catarrhal changes in the tracheal epithelium manifested by the excess of mucus in the lumen produced by increased numbers of mucus-laden goblet cells was seen less often than the same change in smaller airways. The incidence and degree of severity of catarrhal tracheal changes correlated positively with concentration of TNM. In addition, squamous metaplasia was seen as focal lesions in the tracheal epithelium of rats exposed to 5.0 and 7.5 ppm TNM. This change represents a response to a greater level of irritation than that seen in tracheal epithelium with catarrhal exudation alone. Lung edema, characterized by exudation of a protein-rich fluid into the alveolar spaces, was the most severe lesion seen directly attributable to the inhaled TNM. This lesion was seen only once in all control animals. Rats exposed to 3.5 ppm, 5.0 ppm, and 7.5 ppm TNM had lung edema in 7%, 14%, and 75% of each group, respectively. Alteration in the alveolar wall reflected the degree of exudative change seen in the alveoli. The least severe change was a swelling of alveolar epithelial cells. Next in degree of severity was exudation of pulmonary macrophages into the alveoli. Most profound alveolar changes were thickening of alveolar walls because of an excess of either neutrophils or chronic inflammatory cells within the interstitium. There was exudation of fibrin along with edema fluid in the alveoli of the rats exposed to 7.5 ppm TNM. This last lesion results from severe insult to alveolar capillaries.

TABLE 14

INCIDENCE OF RESPIRATORY DISEASE IN RATS EXPOSED TO TNM AND NO_2

	Percent Incidence												
	C	3.5 ppm TNM	14 ppm NO_2	C	5 ppm TNM	20 ppm NO_2	C	7.5 ppm TNM	30 ppm NO_2	C	7.5 ppm TNM	40 ppm NO_2	
Pneumonitis	13	97	83	10	77	85	39	69	76	43	76	73	
Catarrhal Bronchitis and/or Bronchiolitis	5	90	78	0	60	68	1	66	60	12	82	71	Primary Lesions
Catarrhal Tracheitis	0	10	3	0	20	22	4	33	30	2	36	24	
Lung Edema	0	7	0	0	14	0	1	68	26	0	83	84	
Bronchopneumonia	2	22	2	0	34	0	0	45	18	0	29	3	Secondary Lesions
Histiocytic Pneumonia	2	28	3	3	18	23	3	9	18	7	13	23	
Number of Animals	60	60	60	60	65	60	69	85	82	60	72	70	

Lung disease seen as secondary changes to the primary catarrhal lesions and lung edema was observed in a high percentage of rats exposed to TNM. Bronchopneumonia was the most severe of these changes. The incidence of bronchopneumonia was not concentration dependent, but one third of all exposed rats had bronchopneumonia, while only one control rat had the lesion. As well, the incidence of histiocytic pneumonia was increased in exposed rats over that seen in controls. Architectural changes in lung tissue, noticeably emphysema and atelectasis, were seen frequently in rats exposed to TNM. These changes are natural consequences of obliterative disease of airways or presence of a large number of fluid filled alveoli.

C. Respiratory Changes in Rats Exposed to NO_2 (see Table 14)

Pneumonitis identical to that described in rats exposed to TNM was seen in a high percentage of rats exposed to NO_2 (79% of exposed rats vs 27% of controls). This change was not positively concentration dependent and the inexplicable slight reduction of this lesion seen in rats exposed to high concentrations of both TNM and NO_2 is due to the mortality in these high concentration groups. Early death in these animals shortened the time needed for this proliferative lesion to develop. Catarrhal changes in the tracheobronchial tree approximate those seen in rats exposed to TNM. A high percentage of rats exposed to NO_2 had catarrhal changes in the air passages of the lung (69% of NO_2 exposed rats vs 4% of control rats). Rats exposed to 14 ppm NO_2 were largely free of catarrhal tracheitis, but 26% of the 20 ppm, 30 ppm, and 40 ppm NO_2 concentration rats had this lesion. No increase in significant concentration dependent incidence nor degree of severity was noted for the three highest exposure groups. Fine changes in the mucus membrane of the tracheobronchial tree parallel the degree of exudation seen; however, squamous metaplasia of the tracheal epithelium was absent in rats exposed to NO_2. Lung edema was not seen in rats exposed to 14 and 20 ppm NO_2. One fourth of rats exposed to 30 ppm NO_2 had lung edema, and the highest dose group (40 ppm NO_2) had this lesion in 84% of the animals - clearly a concentration dependent response once the effective threshold of injury is achieved. Fine changes in the alveolar walls of rats exposed to NO_2 do not correlate well with the presence of alveolar edema. All concentration groups had alveolar wall thickening and swelling of alveolar epithelial cells. Exudation of alveolar macrophages was noted in the three highest concentration groups. Severe irritation exemplified by exudation of fibrin into the alveoli was only seen in the lungs of rats exposed to 40 ppm NO_2.

Secondary pulmonary disease (bronchopneumonia and histiocytic pneumonia) was seen sporadically in rats exposed to NO_2. A uniform incidence of histiocytic pneumonia was seen in rats exposed to 20 ppm, 30 ppm, and 40 ppm NO_2 (21% of exposed rats vs 4% of controls). Emphysema and atelectasis of lung tissue occurred in connection with either plugged airways or profound lung edema.

D. Comparison of Pulmonary Lesions seen in Rats Exposed to TNM and NO_2

Qualitatively, the pulmonary lesions seen in rats exposed to these concentrations of TNM and NO_2 are identical, and represent the response of the tracheobronchial tree and lung alveoli to irritation of a mild to moderate degree. Severe irritation usually elicits necrotic and diphtheritic changes in mucus membranes plus acute, severe loss of lung capillary integrity resulting in flooding of the lungs with edema, and resultant death prior to any cellular inflammatory response.

A comparison of the respiratory lesions resulting from exposure to 3.5 ppm TNM and 14 ppm NO_2 is remarkably similar. As well, the pulmonary response of rats exposed to 5.0 ppm TNM and 20 ppm NO_2 is similar. Rats exposed to 7.5 ppm TNM had more severe respiratory changes than did those exposed to either 30 ppm or 40 ppm NO_2. These findings are reflected in greater mortality seen in the 7.5 ppm TNM than that seen in either 30 ppm or 40 ppm NO_2.

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